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THE COAGULATION OF BLOOD.

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RECENT investigators of the phenomenon of blood coagulation have come to rather widely divergent conclusions. Morawitz,¹ attempting to harmonize the older views of Schmidt with the later work of Pekelharing, Hammarsten, and others, gives four factors entering into the process. Three of these pre-exist in the circulating blood, — the fibrinogen, the thrombogen or proferment, and the salts of calcium. The fourth factor arises at the moment of coagulation, and is produced by the disintegration of the formed elements of the blood, — the platelets and probably also the leucocytes. This substance is the thrombokinase, which activates the thrombogen in the presence of calcium salts to form thrombin. The thrombin then attacks the fibrinogen molecule and in the manner of a ferment or catalyzer changes it to fibrin. The thrombokinase may also be derived from the various tissues of the body, and when so extracted serves to activate the thrombin and initiate coagulation. The suggestion of a "kinase," as made by Pawlow in the study of the entero-kinase in the intestines, is here applied to explain the action of the "zymoplastic" substances of earlier workers.

On the other hand, Nolf,² impressed by the fact that a clot is soon re-dissolved by an act of autolysis, especially in the case of clots of fibrinogen produced by thrombin, sees in the phenomenon of coagulation, as the paramount question, not what makes blood clot, but what makes the clot persist. According to his view coagulation is a normal process in nutrition and assimilation, whereby the fibrinogen of the blood is rendered available as food for the tissues.

¹ MORAWITZ: *Beiträge zur chemischen Physiologie und Pathologie*, 1904, iv, p. 381. MORAWITZ: *Ergebnisse der Physiologie*, 1905, iv, p. 307; contains a full bibliography.

² NOLF: *Archives internationelles de physiologie*, 1906, iv, p. 165. *Ibid.*, 1908, vi, pp. 1, 115, 306. *Ibid.*, 1909, vii, pp. 281, 380.

Coagulation is normally and continually going on in the blood. It consists of a quantitative and mutual precipitation of two colloids, the thrombin and the fibrinogen. The thrombin is formed by the union of the "hepato-thrombin," which has its source in the liver and corresponds in a way to the thrombogen of Morawitz, and "leuco-thrombin," which is derived from the leucocytes and platelets of the blood. In the circulating blood small amounts of thrombin are formed around the leucocytes in the manner described. This precipitates a small amount of fibrinogen as fibrin, which covers the leucocyte with a thin ultra-microscopic layer of fibrin. This layer of fibrin renders the leucocyte neutral to some extent, in the matter of further production of leuco-thrombin. This precipitated cuticle of fibrin is now attacked by the real enzyme of the thrombin, the "thrombozyme," and dissolved. This autolytic action is a true fermentative process, and serves primarily purposes of tissue nutrition. In abnormal conditions, such as produce ordinary clots, the leucocytes and platelets are destroyed in great numbers, and the excessive amounts of leucothrombin so formed unite with hepato-thrombin to form the active thrombin that produces the clot. The real question here is, what makes the clot persist? This anti-fibrinolytic substance he believes is the hepato-thrombin, to the inhibiting action of which on the fibrinolysis the clot owes its persistence. Coagulation is therefore, according to Nolf, a late phylogenetic adaptation. It is a secondary modification, for preventing hemorrhage, of a primitive nutritive and assimilative process.

Loeb,³ approaching the problem from a study of blood coagulation in the invertebrates, assigns the primary rôle in the process to the *coagulins*. These coagulins may be extracted from the tissues, and when activated with calcium salts have an effect on the fibrinogen similar to ordinary thrombin. These coagulins are not merely zymoplastic substances, nor are they kinases. They do not activate any proferment, but independently attack the fibrinogen molecule. Neither are the coagulins identical with ordinary thrombin. They have a much greater specificity than thrombin, and are more stable.

The fact that tissue extracts properly irrigated will not clot carefully prepared fibrinogen solutions, even when calcium chloride is

³ LOEB, LEO: Archiv für pathologische Anatomie, 1906, clxxxv, p. 160; LOEB: Beiträge zur chemischen Physiologie und Pathologie, 1906, viii, p. 67; *Ibid.*, 1907, ix, p. 185. LOEB: Biochemisches Centralblatt, 1907, vi, pp. 829, 889, excellent bibliography given. LOEB: Beiträge zur chemischen Physiologie und Pathologie, 1909, xvi, p. 157.

added, is explained by assuming that the repeated salting out has changed the character of the original fibrinogen.

Mellanby,⁴ working with the blood of birds, assigns the main rôle in the coagulation of blood to the *kinases*. These kinases, extracted from the tissues, activate the proferment and form fibrin. The proferment seems bound in a way to the fibrinogen, so that the amount of proferment varies directly with the amount of fibrinogen present. As the source of his kinase he used the macerated testes. The fibrinogen solutions were made by diluting the clear bird's plasma with twenty times its volume of water, acidulating, centrifuging, and re-dissolving the precipitate in the original volume of water.

The present investigation was carried on under the direction of Dr. W. H. Howell, to whose stimulating interest and many valuable suggestions most of any credit which this work may have is largely due.

The first requisite in the investigation of blood coagulation is a trustworthy testing solution. The ideal solution would be the circulating blood, if it could be kept in its normal condition. This is of course impossible, at least with mammalian blood, inasmuch as it begins to clot immediately after taking. Recourse must therefore be had to artificial plasmas. It is believed, for reasons to be stated further on, that the most satisfactory testing plasma is a solution of fibrinogen, prepared essentially after the manner of Hammarsten, with two modifications.

The method of preparation of the fibrinogen solutions used in this work was as follows:

On account of the fact that the blood of the cat does not "lake" so readily as that of many other mammals, this animal was used in the majority of experiments. The blood was taken under careful conditions from the carotid artery, through a cannula and short tube either oiled with some neutral oil or washed out with a 1 per cent solution of sodium oxalate. A 1 per cent solution of sodium oxalate was added in the proportion of one part of oxalate solution to nine parts of blood. The blood was then immediately centrifuged, until a clear supernatant plasma was obtained. Sodium chloride was added to a little more than 50 per cent saturation, and the precipitate after centrifuging, and washing in a saturated solution of sodium

⁴ MELLANBY: The journal of physiology, 1909, xxxviii, p. 28.

chloride by decantation, was re-dissolved in a 2 per cent solution of sodium chloride of volume equal to the original plasma.

A second and a third precipitation in the same manner produced a final precipitate, which was dissolved in 0.9 per cent solution of sodium chloride. If such a solution of fibrinogen still remained distinctly opalescent, 1 c.c. of a dilute solution of barium chloride and 1 c.c. of a dilute solution of sodium phosphate were added. The dilution of the two solutions was approximately such that 1 c.c. of the barium chloride and 1 c.c. of the sodium phosphate neutralized each other. The precipitate of barium phosphate so formed gradually fell to the bottom, and with it were swept out the particles giving the opalescence, leaving finally a clear solution of fibrinogen, not very different from the clear appearance of distilled water.

The solution of fibrinogen was then placed in a collodion tube and dialyzed against a large volume of 0.9 per cent solution of sodium chloride for about eighteen hours. This insures a practically complete removal of any oxalate remaining, or of any excess of barium chloride or sodium phosphate. It also insures to the fibrinogen solution a known concentration of sodium chloride.

Such test solutions of fibrinogen do not clot spontaneously, even when kept until putrefactive changes arise. They give no appearance of a clot on the addition of calcium salts, showing that no pro-stage remains in the fluid which can be activated by calcium. No clotting occurs either when Ringer's solution is added, such as occurs frequently in fibrinogen solutions, which have been precipitated only once or twice and which remain strongly opalescent. Probably in such opalescent solutions some of the fibrinogen has already been affected by the thrombin present. It is believed therefore that by this method a solution of fibrinogen was obtained which was entirely free from any substance which could give rise to the thrombin. Only with the aid of such solutions can reliable experiments be made upon the presence or absence of a coagulating agent.

That solutions of fibrinogen prepared as here described are, however, not materially changed from the condition found in the blood is shown by the fact that with serum or thrombin they form a typical clot, which resembles in every essential the normal blood clot. The promptness of the action and its similarity in every way to clots of other forms of test solutions prove clearly that the fibrinogen in question has retained its characteristic properties, and by its purity

and stability is peculiarly fitted to study the real coagulating effects of other factors entering into the process. With this fibrinogen solution the main constituents of normal plasma were tested as to their coagulating properties. As already pointed out in describing the method of preparing fibrinogen, the inorganic salts of the blood in normal (Ringer) concentrations had no effect, either added singly or together or in other possible combinations. Similar negative results were found in the case of solutions of lecithin and cholesterin. Carbon dioxide and oxygen gave negative results. The experiments showed, as has long been known, that the initiating factor of coagulation is not among the simpler chemical substances which exist preformed in the circulating blood.

THE THROMBIN.

The observation that serum added to a solution containing fibrinogen will induce clotting dates back to the work of Buchanan. Later Alexander Schmidt succeeded in getting the active substance in somewhat purer form by treating the serum with alcohol and extracting the dried residue with water. This substance he designated *fibrin ferment*, or *thrombin*.

The thrombin used in these experiments was prepared after Schmidt's method. Blood was drawn from an animal and allowed to clot. As soon as the serum was sufficiently pressed out of the clot, it was diluted with twenty times its volume of alcohol (95 per cent) and allowed to stand for a period ranging from several days to several months. The duration of the action of alcohol seemed to have no very appreciable effect on the quantity or strength of the thrombin extracted. After the proteins had been precipitated by the alcohol they were filtered off, dried, and extracted with water. Into this aqueous extract other things as well as thrombin will enter. Evidently the free salts of the serum will be dissolved. Some of the proteins also go into solution, especially if the aqueous extraction is continued for any considerable length of time. Unfortunately therefore such *thrombin* solutions are not very pure, and offer serious obstacles to a study of the chemical nature of this substance.

If however the extraction is made of short duration, it is possible to secure a thrombin solution which is very active in producing coagulation and which nevertheless gives only the faintest traces of

protein. The excess of salts may be removed by dialyzing the thrombin solution in a collodion tube against pure water. Experiments soon showed, however, that the dissolved proteins interfered in no way with the thrombin action, and some of the strongest thrombin solutions were secured by submitting the dried residue not only to prolonged aqueous extraction, but even to prolonged putrefaction, until the protein residue disappeared. Such solutions usually gained in amount of thrombin, as the processes of putrefaction continued, the possible explanation for which will appear in another connection. It is evident that the thrombin extracted from the serum is of the greatest importance in the process of coagulation. It appears in the plasma at the time of coagulation and is the agent which effects the change from the liquid fibrinogen to the solid fibrin. It seemed desirable therefore to determine the conditions under which it acts, its origin, and as far as possible its chemical nature.

Rapidity of coagulation with thrombin. — The rapidity of coagulation was found to be directly proportional to the amount of thrombin added, as indicated in the following experiment:

20 c.c. fibrinogen + 40 drops thrombin extract,	14 min.
20 c.c. fibrinogen + 20 drops thrombin extract,	26 min.
20 c.c. fibrinogen + 10 drops thrombin extract,	50 min.
20 c.c. fibrinogen + 5 drops thrombin extract,	90 min.

The time required for coagulation was determined by the moment when the glass could be inverted without spilling the contents. Such a rate, however, holds good only below certain limits. If the amounts of thrombin become excessively large, the rapidity of coagulation does not vary in similar proportion. There is soon reached a maximum amount beyond which the further increase of the amount of thrombin no longer increases the rapidity of coagulation. The evident reasons for this appear in a study of the quantitative relations of thrombin and fibrinogen.

Quantitative relations of thrombin and fibrinogen. — The original statement of Alexander Schmidt that minimal amounts of his "fibrin-ferment" always produced complete coagulation was soon questioned. It was found that partial clots could arise, or that a second or even third successive clot might form in the same fibrinogen solution or plasma. Careful quantitative experiments seem, however, not to have been made. For the experiment on this point

500 c.c. of clear plasma of four cats were taken, and from this plasma 500 c.c. of fibrinogen solution were prepared. This amount was divided into four equal parts. To part one 5 drops of a tested thrombin solution were added; to part two, 10 drops; to part three, 20 drops, and to part four, 40 drops. The glasses of fibrinogen were then allowed to stand untouched for twenty-four hours at room temperature, to allow ample time for coagulation. The fibrin from each glass was then collected on a filter, washed, and placed in a weighing tube, which together with the filter had been previously heated several times at a temperature of 110° C. to approximately constant weights. The weighing bottles with the several amounts of fibrin were then heated for a number of hours at 110° C., weighed, and reheated until fairly constant weights resulted. Final weighing gave the following results:

5 drops thrombin . .	0.2046 gm. fibrin.
10 drops thrombin . .	0.3573 gm fibrin.
20 drops thrombin . .	0.6089 gm. fibrin.
40 drops thrombin . .	1.5872 gm. fibrin.

It will be seen, from these results, that while they fall short of exact mathematical proportionality, yet they reveal very clearly that there is in the formation of the fibrin a definite and direct quantitative proportion. The bearing of such facts on the supposed fermentative nature of thrombin is treated farther on. It need not be pointed out that such quantitative relationships will appear only when the amount of thrombin is below the maximum amount required to affect the entire fibrinogen. Adding thrombin beyond this maximum amount can of course be of no moment, there being no further fibrinogen available.

Effects of temperature on coagulation with thrombin.— Similar amounts of fibrinogen and thrombin submitted to different temperatures showed that temperatures between 17° C. and 41° C. had practically no varying influence on the time of coagulation. In one experiment on this point the following results were obtained:

Temperature.	Time of clotting.
1° C.	2 hours
17° C.	28 min.
21° C.	28 min.
31° C.	27 min.
41° C.	29 min.

These results agree very closely with the determinations made by Mellanby on this point. The temperature coefficient of coagulation between the temperatures of 17° C. and 41° C. is practically 1. Whether this is to be interpreted to mean that the process of coagulation is essentially a physical or physico-chemical one, or at least is overshadowed by physical moments, is perhaps an open question. It is well to recall, however, that in the experiments here noted the thrombin was ready formed. The time element in the coagulation, as determined by a coagulometer, on freshly drawn blood is a different matter. In this case there is a definite relation between the temperature and the rapidity of the coagulation, as indicated in a few figures taken from Addis: ⁵

Temperature, ° C.	Coagulation time, min. sec.
10.25	21 30
12.25	16 30
13.5	14 32
16.5	10 10
18.5	7 34
19.5	6 2
20.5	5 22

It is obvious that in these latter experiments additional factors are concerned, which give rise to the thrombin, — either processes of secretion, or disintegration of formed elements, or chemical activation, which drop out when ready-formed thrombin is used.

PROPERTIES OF THROMBIN.

1. Its stability with reference to heat. In Alexander Schmidt's reports of the fibrin-ferment discovered by him, as given in his book "*Zur Blutlehre*," he cites, as one proof of the fermentative nature of the substance, that it is completely destroyed by boiling. A footnote, however, shows that he had overstated this point, inasmuch as he calls attention to the fact that "ordinarily one finds that boiling does not completely destroy the ferment." Howell also notes

⁵ ADDIS: Quarterly journal of experimental physiology, 1908, i, p. 305. ADDIS: Quarterly journal of medicine, 1909, ii, p. 149.

the same fact. In the experiments made upon this point in the present investigation it was found that in aqueous solutions fairly free from proteins the thrombin is not wholly destroyed, and the freer the solution is from proteins, which coagulate on heating, the greater is the amount of thrombin which can bear boiling. Ordinary fresh thrombin solutions, after several minutes of boiling, still cause typical clotting. It is therefore not so thermolabile as often asserted, and its reaction to heat can hardly be cited as proof of its essential similarity to other enzymes.

In dried form it withstands a temperature of at least 135° C. for half an hour. In regaining the thrombin from such dried extracts it is necessary to submit it for several days to the dissolving action of water. The necessity for this no doubt lies in the excessively hard baking to which the mass has been exposed, and the difficulty with which subsequent extraction takes place. The extraction may be facilitated by allowing putrefactive changes to arise, which probably, in a manner to be described later, liberate the thrombin enclosed. The thrombin is, however, completely destroyed when charred or ashed. Extracts after such treatment show no trace of its action. Moreover, thrombin is only slightly diffusible. Placed in a collodion tube, it may be dialyzed against pure water for six or seven hours without serious loss. The conclusion is warranted therefore that thrombin is not a simple inorganic compound.

Recovery of inactivated thrombin.— While aqueous solutions of thrombin withstand, in large part, the boiling temperature, solutions of thrombin containing proteins are at first completely inactivated by that temperature. That the thrombin has, however, not been really destroyed is shown by the fact that its action may be recovered by simple processes. This recovery may be accomplished by submitting the solution of inactivated thrombin to the action of a small amount of sodium hydroxide. Not quite so effective is treatment with a dilute mineral acid for a few minutes. In this way amounts of thrombin may be regained depending upon the maximum temperature used. Instead of the action of the sodium hydroxide or acid, similar results are obtained by submitting the inactivated thrombin solution to putrefactive changes. Such a protein-containing solution of thrombin we have in the serum of the blood. Heating serum to 56° or 57° C. is usually stated to be sufficient to destroy the thrombin present and to insure a thrombin-free serum. This was found not to be strictly true. Active serum may be heated

above 57° C. and its thrombin action regained in part at least. If serum is heated to 65° C. for several minutes, it is found to be inactive when added to a fibrinogen solution. If however such an inactivated serum is treated with a small amount of sodium hydroxide for a few minutes and then neutralized, it will coagulate fibrinogen, whereas a control of heated serum not so activated has no coagulating effect on solutions of fibrinogen.

It is an old observation that serum soon loses its active thrombin on standing, and becomes ineffective in producing a clot. According to older observers the thrombin was supposed to disappear entirely. When later such inactive serum was activated with sodium hydroxide or mineral acids, and a thrombin activity as much as thirty times the original strength of fresh serum was secured, it was explained as being due to a proferment in the blood which had been activated and from which new thrombin had been formed. Such was the β proferment of Morawitz, in distinction from the α proferment which is activated by calcium alone. Later the disappearance of the thrombin from serum was explained as due to the formation of a *metathrombin*, which may be changed back to thrombin by the alkali or acid activation referred to.

The parallelism between the gradual disappearance of thrombin from serum on standing and its recovery by activation, and the disappearance of thrombin from serum on heating to 65° and its recovery by a similar activation, strongly points to the conclusion that these two things are essentially the same phenomenon, and that the disappearance of the thrombin is probably due to its combining in some loose way with some substance, possibly the proteins of the serum, from which it may in turn be liberated by agencies which tend to destroy this combination. That the disappearance of the thrombin is due to such a loose combination with some substance, and that activation by alkalies is due to the breaking apart of this connection, seems probable from the further fact that serum, which has become completely inactive on standing, becomes later, if submitted to putrefactive processes, quite active again. To procure such an active condition by putrefaction often requires a number of days, on account of the remarkable resistance shown by serum to putrefactive changes. Whether this union is of a chemical nature or not, is not easily told. It seems, however, clearly more than mere mechanical adsorption, for neither in the standing serum nor in the serum inactivated by heating to 56° C. is there any precipitation to

make mere mechanical adsorption possible. The process is evidently here more nearly allied to a loose but truly chemical combination.

Thrombin solutions containing proteins, when heated to points above the coagulation points of these proteins, may no doubt carry down the thrombin in a more mechanically adsorbed way.

Experiments make it certain that thrombin does not unite with all proteins. If it is a protein with which it unites, then there is a marked specificity in this respect. If, for instance, serum be allowed to stand, not only will the thrombin originally in it disappear, but it will also inactivate large amounts of thrombin added to it. In some experiments serum was able to neutralize its own volume of a thrombin solution whose activity as tested on fibrinogen was greater than that of fresh serum. If however thrombin is added to a solution of egg-albumen and the mixture is allowed to stand for several days, while its coagulating power is tested from time to time on fibrinogen solutions, it is found that practically none of the thrombin has disappeared, whereas blood serum in a similar experiment loses its coagulating power, unless it is re-activated or is submitted to slight putrefactive changes. If serum be heated to 80° C. *before* the thrombin is added, and the mixture is then allowed to stand, it is found, on testing with fibrinogen solutions, that the thrombin does not completely disappear. Clotting is evident, although not so immediate and complete, if the mixture has stood for several days, as with fresh thrombin solutions. The partial disappearance of the thrombin in this case may be due to the fact that the preliminary heating has only partly destroyed or altered the substance which combines with the thrombin. If this substance consists of the proteins of the blood, we can understand why the serum after being heated to 80° C. still possesses some power to inactivate the thrombin, since it is well known that not all of the protein is coagulated at this temperature.

The view that the union of the thrombin takes place with some blood protein is strengthened further by experiments which showed that thrombin is not neutralized by other known constituents of the blood. Attention was especially directed to cholesterin. Cholesterin solutions, both aqueous and soapy, had however no effect on thrombin solutions after days of standing.

It has been pointed out that the thrombin and fibrinogen unite in quantitatively proportional amounts in the formation of fibrin, and that coagulation in this respect is the mutual precipitation of these

two colloids as suggested by Nolf. But the union seems a relatively loose one. If less thrombin is added to an amount of fibrinogen than is required to precipitate the entire fibrinogen, a partial clot will form. If this clot be removed, no thrombin remains in the fibrinogen solution, and it will remain fluid unless new thrombin is added. But the fibrin removed yields relatively large amounts of thrombin, when "digested" according to the method of Gamgee, in a 5 per cent solution of sodium chloride at 40° C. for several hours. In this "solution of the fibrin" the thrombin is liberated anew. The same result may be reached by submitting the fibrin to putrefactive processes. As the fibrin dissolves, tests with fibrinogen solutions indicate a larger and larger amount of thrombin liberated. The question naturally arises whether, if it is possible to regain the thrombin as thrombin from fibrin, it may not be possible by proper treatment to regain the fibrinogen as fibrinogen.

ISOLATION OF THROMBIN.

In approaching the problem of isolating the thrombin, experiments were made to determine the media which dissolve thrombin and those out of which it is precipitated. It is evidently not dissolved by alcohol. In Schmidt's method for obtaining thrombin it is the dried precipitate after the removal of the alcohol that yields the thrombin. That practically no thrombin is dissolved in the supernatant alcohol is shown by the fact that when the alcohol is slowly evaporated and the residue is extracted with water, no perceptible traces of thrombin are obtained.

It is equally insoluble in ether, in chloroform, and in acetone. The dried alcohol precipitate of serum extracted with these liquids gives no trace of thrombin when the extract is evaporated and re-extracted with water. Aqueous extracts, however, of the dried alcohol precipitate, after having been submitted to the ether, chloroform, or acetone for hours, yield the usual amount of thrombin, showing that none has been dissolved out.

But thrombin is very soluble in aqueous solutions. The dried material which had been precipitated from serum by excess of alcohol, when shaken with twice its volume of distilled water for fifteen seconds only, showed a considerable thrombin activity.

Attempts to extract the thrombin with water soon showed that

not only the free salts of the dried material, but some of the protein as well, went into solution, especially so if the extraction was continued a few hours. The salts are readily removed by dialysis against distilled water, and it was hoped the thrombin could be separated from the excess of proteins by reprecipitating the proteins with alcohol and extracting anew with water. In this manner it was found that the proteins were thrown out in large part in three reprecipitations, but the thrombin activity was greatly reduced, in fact practically destroyed. Whether this is due to the fact that the thrombin was coagulated along with the proteins is not certain. It is possible that the toxic action of alcohol is more marked in purer thrombin solutions, while in solutions containing considerable amounts of blood proteins it is protected in some unknown way. The method of reprecipitation gave in all cases finally negative results.

To reduce the amount of proteins to a minimum, the dried alcohol precipitate of the serum was extracted with water for a period not exceeding one minute. In this short time, as stated, considerable amounts of thrombin were extracted, as shown in tests with fibrinogen solutions. Such solutions of thrombin showed only the faintest traces of protein when treated with the ordinary color tests, and they remained clear on boiling. When evaporated only a small amorphous residue remained.

That thrombin is, however, not a nucleo-protein, as believed by Pekelharing, is indicated by the fact that a thrombin solution may be acidulated with acetic acid, and the nucleo-proteins precipitated. If this precipitate is removed by centrifuging or filtering, and the filtrate at once neutralized, the thrombin activity is not materially reduced. The neutralization may not be postponed too long, as acids and alkalies, if allowed to act sufficiently long, are destructive in their effects.

While this experiment shows that we are not dealing with an ordinary nucleoprotein, it is more difficult to arrive at conclusions concerning the protein nature of thrombin. There are some facts which argue against its protein structure. As pointed out by Schmidt and others, it is possible to secure thrombin solutions which are quite active and which give only the faintest traces of protein. As stated in an earlier part of this paper, it stands boiling, and so differs at least from the ordinary proteins associated with it in the blood.

More significant, however, is the resistance of thrombin to putre-

fective changes. Bottles of thrombin containing large amounts of dissolved proteins were allowed to stand for months, until the putrefactive changes had run their normal course. Such thrombin solutions lost none of their activity, in fact they gained materially in the initial stages of the process. It should be pointed out, however, that such solutions, even after several months of standing, still show very distinct protein reactions, so that it would be entirely wrong to hold that the cleavage due to putrefaction had been complete.

On the other hand, some of the properties of thrombin are similar to those of the proteins. It is not readily diffusible. Dialyzed against pure water, it may, as stated before, be separated from salts present without serious loss. It is, however, slightly diffusible through a collodion membrane, and when allowed to dialyze against a smaller amount of water, it may be shown that distinct traces of thrombin appear on the outside after several hours.

Like proteins, it seems to be broken down by peptic and tryptic digestion. Solutions of thrombin submitted to ordinary peptic (acid) or tryptic (neutral) digestion always lost their coagulating activity. But other possibilities than those of regular peptic or tryptic digestion exist. In the case of the pepsin-hydrochloric experiment it may be that the prolonged action of the acid is toxic. Control experiments seemed to show that this was true. In the case of the tryptic digestion there was the disturbing action of the trypsin itself upon the fibrinogen of the test solution. Relatively small amounts of trypsin (neutral) in fibrinogen solutions induce active lytic effects, before coagulation is effected. In some experiments the mixture of thrombin solution and trypsin after having been left at room temperature for eighteen hours was first heated to 40° C. for two hours, and subsequently in order to destroy the trypsin was heated to 65° C. Then by careful activation an attempt was made to restore the thrombin. But all such attempts led to negative results, and it seems probable that thrombin is destroyed by the digestive action of both pepsin and trypsin. While it is impossible therefore to speak definitively as to whether thrombin is a protein or not, it is clear that it is not a simple inorganic compound. Some of the reactions of thrombin suggest its possible relationship with histones or protamines. Attempts have been made to test experimentally whether relatively pure thrombin solutions give the characteristic histone and protamine reactions, especially the precipitation out of aqueous solution by ammonia. The work on this point has not proceeded far enough to warrant any definite conclusions.

SOURCE OF THROMBIN.

Thrombin exists in serum, but what its antecedents are in the circulating blood is a question that has been variously answered by different investigators. It has been assumed by many that there exists in the circulating blood a proferment or thrombogen or hepato-thrombin, different names by different authors for essentially the same thing. This pro-thrombin Nolf believes is formed in the liver; according to Morawitz it is derived from the formed elements of the blood. Activated by calcium salts, and by a *kinase* derived from leucocytes or tissues, the pro-stage becomes the active thrombin. By others the existence of a definite thrombogen or proferment in normal blood is denied. A series of experiments was undertaken to determine the existence or non-existence of such a body. It was necessary in these experiments to treat the blood in such a manner, at the very moment of taking, as to prevent the usual changes which occur at that instant and which usher in coagulation. The procedure consisted in laying bare the carotid artery of the cat and inserting a cannula. The cannula and short tube were thoroughly oiled with a neutral sterile oil. On opening the artery the first few cubic centimetres of blood were rejected. About 50 c.c. of the blood were then led into a large jar of 95 per cent alcohol. Vigorous stirring of the alcohol brought the small stream of submerged blood into general and instantaneous contact with the greatly excessive amount of alcohol and so insured immediate precipitation and fixation. The remaining amount of blood drawn from the artery (50 c.c.) was led into a beaker and allowed to clot. The serum from this clot was precipitated with twenty times its volume of alcohol. It was thus possible to have two parts of blood from the same animal, — one part precipitated by alcohol, before any coagulative changes occurred, the other part after the process of coagulation had run its course. The two precipitates were then dried, and aqueous extracts were made in the usual way for preparing thrombin solutions. Such an extract from the serum showed, of course, the usual thrombin activity. Extracts from the blood, taken immediately under alcohol, showed *no traces* of thrombin. Tested with fibrinogen solutions, no evidence of clotting of any kind appeared. Many similar experiments by Schmidt and others had shown that, with greater and greater care in taking blood under

alcohol, less and less evidence of "ferment" appeared. It is certain, therefore, that in circulating blood no appreciable amounts of *ready-formed* thrombin exist. *Does a proferment or thrombogen exist?* To determine this, if possible, extracts of the blood caught immediately under alcohol were activated with dilute sodium hydroxide and dilute mineral acids without giving any traces of thrombin. Activation with calcium salts in varying proportion proved equally negative. No evidence of any kind could be found that there existed in the blood extract any substance which could be activated by calcium salts, alkalies, or acids to real thrombin activity.

But, as stated above, those holding to the view that a thrombogen exists in circulating blood assert that the activation is by a kinase in the presence of calcium salts. Extracts of the alcohol blood were therefore made and calcium salts were added in proper proportion. Assuming that a prothrombin was present in the circulating blood, these extracts lacked only the kinase to produce active thrombin. This kinase, according to Morawitz, Nolf, Mellanby, and others, may come from the tissues. Extracts of tissues which had been carefully irrigated with a Ringer's solution were added to the alcohol-blood extract, and after standing for varying lengths of time the coagulating power of the mixture was tested with fibrinogen solutions. In no case was any evidence of thrombin action observed, in spite of the fact that all the supposed elements entering into the formation of active thrombin were present. If it be urged that the treatment with alcohol has destroyed the proferment, it need only be recalled that in the preparation of alcohol plasmas by the alcohol-precipitation method, authors assert the existence of the proferment unimpaired.⁴ It seems probable from such results that thrombogen does not exist in circulating blood. In fact, in reading the literature on this point, one is impressed with the fact that the reasons for the existence of a thrombogen in circulating blood rest more upon speculative considerations than upon experimental evidence. It would seem that the normal existence of such a body, whose assumed presence in quantity certainly tends to complicate the interpretation of the process of coagulation, should be granted only when definite experimental evidence makes such an assumption necessary. No such necessity exists to-day, and thrombogen as a normal constituent of blood is a theoretical rather than a demonstrable thing. But thrombin does not exist as such in plasma. It must have material antecedents from which it is produced. The

conclusion seems probable that it has its source under abnormal conditions in greatly accelerated processes which affect the leucocytes or platelets of the blood. Whether the substance formed is a result of secretory activity or a product of cell disintegration, it is impossible to say. But a number of facts point unmistakably to the formed elements of the blood as the source of the pro-stage. Plasma in which the corpuscles have been removed immediately after taking has a greatly increased resistance to coagulation in avian and reptilian blood. In centrifuged blood experiments showed that parts of the oxalated plasma taken from the lower part of the tube near the layer of corpuscles yielded a much more active thrombin solution than a similar amount taken from the top of the tube, where the plasma was much freer from corpuscles. Substances in actual solution in the plasma could scarcely have shown such an unequal distribution. The absence of thrombin or its pro-stage in the blood taken under alcohol as just described finds a ready explanation in the assumption that the corpuscles were in this case coagulated by the action of the alcohol before the normal processes of disintegration were possible. Whether the substance so formed is of an ordinary protein or histone or protamine character, or is a still simpler organic substance, is undetermined, but no matter what its nature may be it is unable to produce the normal formation of fibrin unless activated by calcium salts. It is possible that other as yet undetermined conditions assist in this activation. This activation may find its simplest explanation in assuming that the calcium enters chemically into the thrombin molecule, as suggested by Melanby, and by Loeb for the coagulins. If therefore the free calcium is thrown out of the blood by chemical agents, it remains fluid because the prothrombin becomes an active coagulating agent only when combined with this minimal amount of calcium, for the rôle of which strontium alone may be substituted with any success.

Sodium-oxalate plasma. — The fluidity of oxalated blood is, as has often been pointed out, due to the precipitation of the calcium. It is not due to a mere inhibition exercised by the oxalate. This was shown by taking oxalated plasma and dialyzing the same in a collodion tube against 0.9 per cent solution of sodium chloride for hours. In this way any excess of oxalate is removed, as may be shown by chemical tests. Centrifuging will remove any precipitate, and a fluid plasma is thus obtained which shows no tendency to coagulate when allowed to stand for days. Additions of very slight amounts

of calcium chloride bring about a speedy clotting, showing that the prothrombin is lacking only in that salt. A point of interest is in the observed fact that such plasma solutions may stand for several days, and yet give immediate clotting upon addition of calcium chloride. The pro-stage of the thrombin does not disappear from the plasma readily. In serum solutions, however, where the activated thrombin is present, this latter disappears very rapidly, as was pointed out in an earlier part of this paper, possibly by combining with some specific proteins in the serum. The thrombin before activation with calcium salts seems not yet to have such combining properties.

The fluoride plasma. — The action of fluorides in preventing the coagulation of the blood has been variously interpreted by different authors. Thus, according to Arthus, to whom we are indebted for the introduction of this method, the calcium salts of the blood are precipitated. In this respect it acts like the alkali oxalates. But, unlike the oxalates, Arthus holds that fluoride prevents the formation of a proferment, because calcium salts in excess do not coagulate fluoride blood. This inhibiting action was believed to be due to a certain fixation of the formed elements of the blood. Bordet and Gengou, noticing the precipitate which is produced when calcium salts are added to fluoride plasma, held that the prothrombin together with some fibrinogen was thrown out of the solution and thus caused the plasma to remain fluid. Mellanby attributes his failure to have the fluoride blood clot with excess of calcium to the precipitation of the fibrinogen when calcium salts are added to fluoride plasma. Nolf considers the fluoride blood a definite indicator, not only for thrombin, but for the presence or absence of his thrombozyme. Fluoride blood does not clot, according to Nolf, because there is no thrombozyme present, its formation having been prevented by the action of the fluoride presumably on the leucocytes and platelets.

In the following experiments it will be shown that none of the foregoing interpretations is entirely correct, but that the action of the fluoride is primarily identical with the action of the oxalate.

If a solution of fibrinogen is made from fluoride plasma by precipitating the fibrinogen several times with a 50 per cent saturated solution of sodium chloride, according to the method described earlier in this paper, and this fibrinogen solution is dialyzed in a collodion tube against an excess of 0.9 per cent sodium chloride solu-

tion, the fibrinogen clots. The clot is firm and typical and the "serum" expressed is peculiarly rich in thrombin. This makes it improbable that the calcium salts are definitely precipitated out of the solution. It is further evident that the prothrombin is present in a fluoride plasma. The prothrombin remains inactivated because the calcium is bound in a peculiar way by the fluoride. This loose connection is broken in the dialysis, the calcium thus freed activates the prothrombin, and active thrombin results. As this occurs in a clear plasma, it is not due to secondary changes of the leucocytes, brought about by the dialysis. It is possible that the calcium and the fluoride are bound to some protein of the plasma in such a way that the calcium activation of the proferment is prevented, and yet loosely enough to be dissociated by the dialysis.

If a solution of calcium chloride, of a concentration of 1 gm. anhydrous calcium chloride to 25 c.c. of water, be added to a fluoride plasma, a copious precipitate appears, which is largely composed of protein material thrown down together with the calcium fluoride. If the calcium chloride solution be added carefully, drop by drop, until the point is reached when the addition of a small drop produces no further precipitate, it will be found that the fluoride plasma so treated clots promptly and normally. Such results show that the statements usually made that calcium salts added in excess to fluoride plasma produce no coagulation are not strictly correct. If in the manner described the fluoride is precipitated by the calcium chloride solution and a slight excess of calcium salts remains, the clotting is similar in every way to that produced in oxalate solutions under similar treatment. Which of the proteins of the plasma are thrown down with the calcium fluoride precipitate has not yet been determined, but it is certainly not the fibrinogen which is primarily precipitated. This is evident from the fact that fluoride plasmas still clot, normally after the heavy precipitate which forms on addition of calcium chloride is removed. To test more directly whether fibrinogen suffers any material loss, sodium fluoride was added to a fibrinogen solution to a proportion similar to that in fluoride plasma. Calcium chloride of the concentration described gave, when added, only a slight precipitate, even after standing several hours. Addition of thrombin to the fibrinogen solution so treated with sodium fluoride and calcium chloride clotted in a manner similar to the control of pure fibrinogen. It is probable, therefore, that the protein precipitate in the experiment is not to any considerable ex-

tent fibrinogen. If however the calcium chloride is in excess beyond a certain point, the coagulation is prevented, and if the excess be considerable, thrombin added to the fluoride plasma remains ineffective. The inhibition is therefore of the formed thrombin itself. That it is the great excess of the calcium chloride which inhibits the thrombin is shown in the further experiment that when sodium fluoride is added to remove the extra excess it brings about a prompt clotting.

It would seem therefore clear that the action of the fluoride is essentially the same as that of the oxalate, with the difference that the fluoride is bound in some way with the calcium and protein and remains in solution in ordinary fluoride plasma. The inability of the calcium so bound to activate the proferment present as in oxalated blood explains the fluidity of the plasma. That the fluoride cannot bind ready-formed thrombin is shown by the fact that such ready-formed thrombin added to fluoride blood produces a normal coagulation. Dialysis breaks the loose combination, liberates the calcium, activation of the proferment follows, and thrombin results.

On the other hand, the addition of a solution of calcium chloride of the concentration given to a fluoride plasma precipitates this fluoride-calcium-protein compound, leaving the proferment and the fibrinogen practically unaltered. A slight excess then of calcium salts suffices to activate the proferment, and the resultant thrombin clots the fibrinogen. Excess of calcium salts, however, beyond a certain point inhibits and finally prevents the action of the thrombin formed. Removal of the excess makes clotting again possible.

The essential identity in the action of oxalate and fluoride plasmas not only explains both as due to their action on the calcium, but it renders evident the fact that fluoride plasma cannot be used either as an indicator for thrombin or for thrombozyme, and inferences drawn from its use in this way must remain untrustworthy.

DO THE TISSUES YIELD A COAGULATING AGENT?

One of the most involved chapters in the study of blood coagulation has been the question, whether there are derived from the tissues, as extracts or otherwise, substances which either clot the blood directly, as thrombin does, or which exert a favorable and accelerating influence in the process. Schmidt's "zymoplastic sub-

stances" were supposed by this investigator to greatly facilitate, if not to make possible, the ordinary process of clotting. The work of Hammarsten made clear, however, that coagulation was a property belonging to the fibrinogen and thrombin alone, and that Schmidt's zymoplastic substances were not necessary to produce a normal clot. Perhaps this fact induced investigators who saw a distinctly favorable influence exerted by the extracts of tissues to interpret this effect, not from the side of fibrinogen, but rather from the side of the thrombin, and the effects were ascribed to tissue thrombins. This is the view of Nolf, who holds that, in addition to the leuco-thrombin of the blood, the tissues produce histo-thrombins. The extract from the walls of blood vessels he designates as vaso-thrombin. These views are essentially the same as those of Morawitz, who designates the active substance in the tissue extracts as thrombo-kinase. They do not directly induce clotting, but serve to activate the proferment in the blood. This is further the view of Mellanby, who in his experiments on coagulation used the macerated testes of the cockerel as his source of "kinase." This "kinase" is held to activate the proferment of the blood and so produce the active thrombin which clots the fibrinogen. If this view differs at all from the views of Morawitz, it is perhaps in the emphasis it lays on the kinase.

On the other hand, Loeb, approaching the question from the study of coagulation in invertebrate blood, sees in the extracts of tissues, not kinases, but definite "coagulins" which are able to attack the fibrinogen molecule directly and form fibrin. These coagulins are not identical with thrombin, but have several well-marked characteristics, especially that of their specificity. The question as to the existence of such "kinases" or "coagulins" or "zymoplastic substances" was submitted to examination in the following experiments:

Fibrinogen solutions were made from the blood of an animal in the manner already described. The organ or tissue from the same animal, which was to be studied for the presence of active coagulating substances, was then irrigated by insertion of a cannula in its main artery and the ligaturing of neighboring arteries. The fluid used was Ringer's solution (NaCl , 0.9 per cent; CaCl_2 , 0.023 per cent; KCl , 0.03 per cent; NaHCO_3 , 0.024), with which the irrigation was continued for several hours. In this way the organ or tissue was thoroughly freed from its contained blood. The pro-

portion of salts was but slightly disturbed, and the tissue cells were not seriously modified. That such is the case is fairly evident from the fact that the mammalian heart, so irrigated, beats for hours. There is therefore no reason to believe that the careful and prolonged irrigation has materially changed the tissues. Muscles, for instance, that still contract have also probably retained the power to produce a kinase or coagulin, if such exist. Moreover, it is not probable that any such "thrombic" substance, if it exists ready-formed in the cells of the tissue, would be washed out by the irrigation, for experiments have shown that thrombin, and so probably similarly acting substances, are only slightly diffusible. After such prolonged irrigation, to remove every trace of blood or serum, bits of the organ or tissue were macerated in 0.9 per cent solution of sodium chloride, and portions of this macerated tissue were put into fibrinogen solutions. Of another part of the irrigated tissue filtered saline extracts were made, and these were tested from time to time on fibrinogen solutions. In not a single case in the repeated series of experiments was there found any evidence of coagulation. All such fibrinogen solutions remained fluid, like the controls beside them, while the addition of thrombin induced the ordinary clotting at once. The sweeping statement just made needs a slight modification. In the case of two tissues the results were not completely negative. One of these was the macerated red marrow of bone. This extract produced a slight coagulum over its surface, which did not however extend very far into the solution, and there never formed anything approaching a typical clot. Whether this is to be interpreted to mean that there exists in red bone marrow a source of thrombin is open to question. The marrow remained red after the irrigation, and its extracts were slightly discolored by the hemoglobin. It is possible, remembering the finer structure of this tissue, that perfect irrigation under the circumstances was impossible and that the tissue still contained blood or serum. The other tissue was the spleen. Macerated and extracted, it produced in fibrinogen solutions small flocculi, which soon settled to the bottom as a white precipitate. There was no evidence of clotting. With these two qualifying statements the experiments in every case gave not the slightest evidence of the presence of any substance inducing coagulation. There were, then, evidently no thrombins or coagulins present. Were "kinases" present? These would require prothrombin and calcium salts to produce an active thrombin. The prothrombin

being supposed to exist in circulating blood, it ought to be extracted when blood taken immediately under alcohol and dried is subjected to the dissolving action of water. It has been pointed out that the supposed prothrombin in alcohol plasmas has been procured in essentially the same way. Such extracts when combined with calcium and extracts of tissues thoroughly irrigated gave no evidence whatever of the presence of a coagulating agent. Evidently in the chain of argument as usually presented there is a missing link. Either the prothrombin of the circulating blood or the kinase, or perhaps both, are non-existent. Thus throughout these experiments no hint of a coagulin or a kinase could be discovered. They left unexplained, however, the obvious facts that some forms of blood or plasma do react immediately and definitely to tissue extracts. The experiments on the blood of terrapins and birds noted by previous observers were repeated. Terrapin's blood taken carefully through an oiled cannula and put into a perfectly clean beaker will remain fluid for days. The blood may be centrifuged, and the clear supernatant plasma is then equally or more resistant to spontaneous clotting. If however tissue extracts or pieces of tissue be added, the coagulation is pronounced and immediate. The most apparent explanation is that of a thrombin or coagulin or kinase present in the extract. But this blood or plasma can be made to clot equally well and equally rapidly in ways which preclude the presence of such a definite agent. If, for instance, dust particles, loose sweepings, or linty shreds be added, the coagulation is equally prompt, and in a number of experiments was more rapid than with tissue extract. Boiling the tissue extracts was found not to decrease the coagulating action in the least. Tissue extracts of terrapin were dialyzed against distilled water for twenty-four hours. In this way not only were the free salts removed, but much of the dissolved protein (globulin) was precipitated and the solution seemed relatively much clearer. Such solutions showed a very much diminished effect in producing clotting. It is therefore hard to escape the conclusion that simple physical factors take part in facilitating the coagulation of the terrapin's blood.

Similar experiments were made on birds. The blood was taken, under precautions against foreign contamination, from the femoral artery of a chicken. Such blood, as pointed out by Delezenne, remains fluid for a long time. Centrifuged, it gives a clear plasma which will stand uncoagulated for days. If to such blood tissue

extracts of either the bird or the terrapin be added, clotting as in the case of terrapin's blood is immediate, and the clot is firm. If the clear plasma is taken, the clotting is not quite so rapid. It is held that these tissue extracts contain a "kinase" which activates the prothrombin to thrombin. But the bird's blood or plasma clots with practically the same rapidity and firmness if dust particles are generously added. Bits of down-feathers introduced bring about a speedy clotting. Surely there can be no question of a "kinase" in these instances. Ringer's solution hastens the coagulation time. On the other hand, macerated testis when dialyzed against distilled water for twenty-four hours and then filtered gives a solution which has lost much of its accelerating property. Whether this loss is due to the absence of the mineral salts in the extract, or to the precipitation and removal of the cellular debris from the same, is not clear. Probably both factors enter into the result. Boiling the testicular extract, or other tissue extracts, had no effect, showing that the accelerating substance is not in the least thermolabile, and so differs very greatly from thrombin, which boiled in protein solutions suffers a marked diminution in strength. Mellanby states that he could find no kinase in the muscular tissue of birds. In my experiments in which the fresh plasma of the bird was used muscle extract was practically as effective as extract of testes. In Mellanby's experiments the fibrinogen had been prepared by dilution of plasma with water, acidulation, the centrifuging of the precipitate, and its final re-solution *in toto* in water. Such solutions clotted slowly with calcium salts alone, and very rapidly with extract of testes. Extract of muscle, however, no longer sufficed to produce clotting. It is difficult to escape the conclusion that his fibrinogen solution still contained large amounts of all the coagulating factors of the fresh plasma and showed essentially the same reactions. Remembering the ease with which bird's blood clots on the addition of a great number of entirely foreign substances, and that the rapidity and firmness of the clotting is not materially below that produced by tissue extracts, further investigation would seem necessary before the effect caused by tissue extracts can be attributed to a specific kinase. The supposed absence of such a kinase in muscular tissue, where it would appear to be of the greatest significance and value, if kinases play the important rôle assigned to them, is not easily understood. It has been suggested that the resistance of the blood of bird and reptile to coagulation may be due to the relatively greater

difficulty with which the formed elements disintegrate, and that the explanation of the action of extracts, especially those containing cellular detritus, and of dust particles, lies in the fact that these facilitate the breaking down of the corpuscles. On the other hand, it may be that the foreign substances added form simply "nuclear" points which by some physico-chemical reaction facilitate or initiate the process of clotting. That the tissue extracts of bird or terrapin do not contain an active coagulating agent at all similar to thrombin is shown by the following experiment: Extracts of carefully irrigated tissues of bird or terrapin when added to fibrinogen solutions prepared from cat's blood show no coagulating property whatever, while the fresh serum of bird or terrapin when added causes a prompt clotting, showing that in the latter the thrombin is present in its usual form. This experiment also calls attention to the fact that tissues not thoroughly irrigated will of course show clotting properties, in direct proportion to the amount of blood remaining, and that extracts made from such unirrigated tissues must give untrustworthy results.

ANTI-THROMBINS.

The question whether or not anti-thrombins exist in the blood has been variously answered by different observers. In this series of experiments only one clear evidence of anti-thrombic action was met. This was the neutralizing effect of serum. Fresh serum soon becomes inactive on standing, and relatively large quantities of a strong thrombin solution added to such a serum disappear entirely in a short time. The thrombin may be recovered, in part at least, by proper alkali or acid activation. There is no reason why a similar neutralizing action might not be exercised by the circulating plasma, and in this manner the small amounts of thrombin which probably are being formed all the time may be destroyed. Direct experimental evidence on this point is lacking. The argument of Morawitz, for the existence of an anti-thrombin in blood, because very small amounts of thrombin produce only partial clots, which do not proceed farther, in oxalate or fluoride blood, is to be differently interpreted. This partial clotting is due to the quantitative relations which hold between thrombin and fibrinogen.

Bordet and Gengou, following the general methods of preparing immune sera, injected rabbit serum into the guinea-pig, and so pro-

duced a serum which retarded or destroyed the coagulating action of rabbit serum. This anti-action was upon the thrombin and not upon the fibrinogen. They found that when fresh rabbit serum was added to the fresh serum of the guinea-pig, this latter serum having been previously heated to 58° C. and this mixture was added to the bird's plasma, the latter clotted. If however the serum of a guinea-pig, previously injected as described, was heated to 58° C. and then added to the serum of the rabbit, and this mixture added to bird's plasma, there was no clotting. Accordingly it was assumed that there existed in this immune serum a specific anti-body, not destroyed by a temperature of 58° C., which had inhibited the action of the thrombin in the rabbit's serum.

The immune serum when not heated will, however, at once clot bird's plasma. The anti-body is therefore not effective against the thrombin of its own serum. Whether we are dealing here with the formation of a real anti-body, or whether the results are more involved and due to the play of other factors, further investigation must show. As Morawitz states, the experiments are not fully conclusive and admit of different interpretation. With the more recent production of various specific anti-bodies, such for instance as the anti-chymosin of Morgenroth, it seemed desirable to repeat the work on the possible production of an anti-thrombin which should be effective for the animal's own blood. The presence of such an anti-thrombin in excess would be indicated by a condition of hæmophilia. In the first experiment a medium-sized dog was taken, and the time of coagulation of its blood was determined, by taking a small amount from the vein of the ear. Subcutaneous injections of a thrombin solution were then made every other day, in doses which beginning with 2 c.c. were increased 2 c.c. each succeeding time. The thrombin solution was made by extracting the heated dried alcohol precipitate of serum with distilled water. To insure an active solution, the thrombin was tested on solutions of fibrinogen. After such injections, covering a period of two weeks, the animal was killed. The blood clotted in the normal time, and the serum, when tried on fibrinogen solutions, was found to have the usual coagulating strength. There was therefore no evidence of any kind of the existence of an anti-thrombin. Nor was there any evidence of intravascular coagulation.

In the second experiment a rabbit was taken and active thrombin was injected subcutaneously in amounts which beginning with 2 c.c.

were gradually increased to 20 c.c. The coagulation time of the blood was determined from time to time. It was found that the injection of the thrombin was entirely without effect. There was no evidence of hæmophilia, and the serum after clotting contained the usual amount of thrombin. These results seem to indicate that the organism cannot produce an anti-body for thrombin, or, if it does, the anti-body is not produced in the great excess usual with antibodies. It has been pointed out, however, in an earlier part of this paper that fresh serum is able to neutralize not only its own active thrombin but added excessive amounts. The neutralization is believed to be due to a loose combination with some element of the blood, most probably the proteins. It is possible that the disappearance of thrombin from the circulating blood may be due to the same action. The observed fact that anti-bodies cannot be artificially produced by the injection of relatively simple organic substances, but are limited to substances which are essentially protein, may mean that the thrombin is of the former class, although this observation unsupported by other evidence would be inconclusive as to its non-protein structure.

GENERAL CONCLUSIONS.

The experiments here reported point to the following general conclusions:

1. The coagulation of blood is probably not a fermentative process. Definite statement on this point is complicated by the present unsatisfactory knowledge of the exact nature of ferments and their action. As usually understood, however, ferments act in the manner of a catalytic agent, and they are thermolabile. The coagulation of blood, on the contrary, is, in the first place, clearly a quantitative reaction, and is probably, as stated by Nolf, a mutual precipitation of two colloids, fibrinogen and thrombin. The partial clots which never proceed farther, the successive clots which may be formed by successive additions of definite amounts of thrombin, and the approximate equality in amount between the sum of these successive clots and a maximum initial clot, indicate a definite quantitative proportionality. Careful quantitative weighings of the fibrin formed, as reported in the earlier part of the paper, showed such a direct proportionality between fibrinogen and thrombin as would be required in a definite chemical combination of two substances.

The further resistance of the thrombin in aqueous solution relatively free from dissolved proteins to boiling temperatures is a property not usual with the ordinary catalytic ferments. The recovery of thrombin from protein solutions, such as serum, which have become inactive on standing, or by being heated to 60° C., points toward the same conclusion. Such a recovery, whether by activation with alkali or acid or by putrefactive changes, is believed to depend upon the dissociation of the combination of thrombin with some protein other than fibrinogen. In a similar way the partial recovery of thrombin from fibrin, according to Gamgee's method, is more than the solution of mechanically adsorbed amounts. The three main reasons given by Schmidt originally as proofs of the truly fermentative nature of his ferment are as follows:

(a) Minimal amounts of "fibrin-ferment" will produce a complete coagulation.

(b) The artificial serum contains after coagulation unused ferment with which several successive clots may be produced.

(c) The ferment is destroyed at 100° C.

The first point has been shown to be incorrect. Minimal amounts produce correspondingly small clots. The quantitative relationship holds with all submaximal amounts of thrombin added to a fibrinogen solution. Nor is it possible to find unused thrombin in a fibrinogen solution if a submaximal amount has been added. If the clots are removed after four to twenty-four hours, the remaining fibrinogen solution remains fluid, but clots promptly again on addition of further amounts of thrombin. The probabilities are therefore that the action of the thrombin is in no way that of an ordinary catalytic agent, but that the formation of the fibrinogen is essentially the mutual precipitation of two colloids from the plasma.

2. It has been impossible to find experimental evidence for the assumption that there exists preformed in the blood a thrombogen or proferment.

3. The existence of "kinase" or "coagulins" in the various tissues is improbable. Using carefully prepared fibrinogen solutions, extracts of tissues, irrigated to remove every trace of thrombin-containing blood, cause no clotting. When the addition of such extracts produces coagulation in bloods of bird or reptile, similar results can be secured by the addition of substances such as dust, lint, shreds, etc., which preclude the presence of definite coagulating agents. These results render very probable the assumption that in

such plasmas all the factors of coagulation are in reality present, and the addition of tissue extract or other foreign substance brings into the mixture nothing that can be regarded as a coagulin or as a kinase.

4. The active coagulating agent is thrombin, whose properties and reactions were the chief questions of inquiry in this work. It is derived from the formed elements, in all probability, at the moment of their rapid disintegration when placed under abnormal conditions. In this pro-stage it is inactive in the blood. To become an active precipitating agent it is believed that minimal amounts of calcium salts enter chemically into the molecular complex. Such calcium salts exist in the normal plasma, and the action of sodium oxalate depends on the precipitation of the calcium out of the solution. The action of sodium fluoride consists in preventing the combination of the calcium with the pro-stage of the thrombin, the calcium being bound but not actually precipitated.

5. An anti-thrombin cannot be produced artificially by subcutaneous injection of active thrombin, but the serum contains substances, possibly the proteins, which gradually neutralize the thrombin even when formed in excessive amounts, and this property is perhaps important in maintaining the fluidity of the circulating blood.

6. On the basis of the work here presented it is not necessary to assume the existence of a *kinase* in explaining the clotting of shed blood. The prothrombin formed from the platelets and leucocytes by secretion or by processes of disintegration is activated to thrombin by the calcium salts present, and the thrombin so formed combines in quantitative fashion with the fibrinogen to form fibrin.

7. The proferment found in the blood under abnormal conditions is not readily neutralized. It remains in the calcium-free serum for days, and when activated forms thrombin promptly. Thrombin, on the other hand, disappears very quickly from active serum unless it is re-activated. The ability of the thrombin to unite with other substances of the blood arises only after the calcium activation of the prothrombin.

8. The action of oxalates and fluorides in preventing coagulation is primarily the same. Both salts act by removing the calcium. In the oxalated plasma the free calcium is definitely precipitated, in the fluoride plasma the free calcium remains *bound* in solution. Dialysis or extreme dilution breaks the loose connection and thrombin results. Excess of calcium salts clots oxalated solutions at once. Excess of

calcium salts of sufficient concentration clots fluoride plasmas, after the fluoride-calcium-protein combination is precipitated by this method. In both plasmas great excess of calcium salts inhibits and finally prevents coagulation, although it would seem that fluoride plasma is in this respect more readily inhibited than the oxalate plasma, — a difference which may result from the formation of the heavy precipitate in the fluoride blood.

V I T A

LOUIS JOHN RETTGER was born at Huntingburg, Indiana, October 19, 1867. He received his early training in the common schools of the state. In 1883 he entered the Indiana State Normal School, graduating in 1886. In the fall of that year he entered the Johns Hopkins University, receiving the A. B. degree in June, 1888. He was assistant in the Biological Laboratory of the Johns Hopkins University the following year. In 1890 he was appointed instructor at Indiana University, receiving the degree of M. A. from that Institution in June, 1891. In 1891 he was elected Professor of Physiology in the Indiana State Normal School, which position he still holds. During the winter semester of 1895-96 he attended the University of Heidelberg, and during the summer semester of 1896 was enrolled as a student at the University of Berlin. In 1908 he returned to the Johns Hopkins University, pursuing courses in Physiology, Botany and Zoology.

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